External Effect of Combined Nitrogen on Nodulation [1, 2]

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The many theories advanced to explain the effect of combined nitrogen in nodule inhibition were reviewed by Allison and Ludwig (1) in 1934, and on the basis of their work and that of Mazé (11) and Weber (27) they stated as follows: ". . . that decreased nodulation in the presence of soluble nitrogenous salts is due to inadequate carbohydrate supply in the roots. . . . When nitrogen is abundant, the carbohydrate synthesized is used for top growth, and little is available for growth of roots or nodules." This theory has been generally accepted. Nodulation of plants grown on low soil nitrogen was decreased by weekly spraying with 0.5 % or 1 % solutions of urea (2). The theory is physiologically sound (10, p 201). However, changes in nitrogen level or form can often provide results which are difficult to interpret (8, 12, 15) by using the carbohydrate: nitrogen ratio (C: N) theory (6).

There is some evidence which supports a local or external effect of combined nitrogen. In experiments employing a divided root technique several investigators (5, 7) have shown that nitrate supplied to one part of the root had no influence on infection elsewhere. These observations appeared to be in disagreement with the C: N ratio theory, for if there was sufficient nitrate in the media to completely inhibit nodulation on one-half of the root, there should have been too little carbohydrate available for nodule growth on the other half. Raggio et al. (14), working with excised bean roots, found nitrate inhibited nodulation if supplied in the medium containing root and bacteria but not if supplied through the base of the excised root. They concluded that this questions the theory of an internal effect of nitrogen and suggests an external effect.

Thornton (23) observed a great reduction in both root hair curling and infection threads within root hairs when combined nitrogen was in the medium. Nutman (13) has stated that, with few exceptions, only curled root hairs become infected. It has been suggested that IAA is involved in nodulation, but the mechanism of such an effect is not clear (9, 13, 16, 20, 21, 22).

Although there is evidence for a local effect of combined nitrogen on nodulation, few attempts have been made to propose a mechanism. Virtanen (26) has suggested that nitrate inhibition occurs through the formation of nitrite which reacts to form a nitrite-leghemoglobin complex. Thornton (23) considered that the carbohydrate level in the piliferous layer of the root was important but did not expand on this idea. Thornton and Nicol (24) suggested that the local effect is due to nitrate inhibition of the preliminary curling reaction of root hairs. However, this should be regarded as an observation rather than a mechanism, but merits further study. Cheniae and Evans (3) demonstrated that rhizobia from soybean nodules could produce nitrate reductase. Tonhazy and Pelczar (25) demonstrated that nitrite catalytically destroyed IAA.

Materials and Methods

Four strains of *Rhizobium* were used in this study: *R. japonicum* Kirchner (strains 117 and 123), *R. meliloti* Dangeard (strain Su 388), and *R. Trifolii* Dangeard (strain 205). Antisera were available for checking the purity of 3 of the strains. Rhizobia were grown on agar slants of media containing minerals, 1 % mannitol and 0.5 % yeast extract. Cells were washed from the slants with 1 % sterile mannitol. Unless otherwise indicated the reaction mixtures consisted of approximately 10^6 cells per ml, 1 % mannitol, 10^{-3} M DL-tryptophan, 10^{-2} M KNO_3 with a total volume of 150 ml in a 250 ml Erlenmeyer flask. The pH was adjusted and remained between 6.5 and 7.0. The flasks were stoppered with cotton and set in the dark at room temperature unless otherwise stated. Aliquots were removed at various intervals and cells were removed by centrifugation. Supernatant liquids were used for analytical determinations. Absolute sterility was not maintained in the reaction mixtures. For determination of IAA, 2 drops of concentrated HCl were added to 5 ml of supernatant liquid and the solution was then extracted twice with equal volumes of absolute ether (analytical grade). The ether was evaporated and the residue was dissolved in 3 ml of distilled water. The addition of 1.5 ml of 0.05 M $FeCl_3$ in 35 % $HC10_4$ (Salkowski reagent) resulted in a red color characteristic of IAA (18). The optical density at 525 mμ and the shape of the spectrum were determined by using a Bausch and Lomb Spectronic 505 spectrophotometer. The

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density values were converted to molar concentrations from a standard curve and reported as μmoles/ml of reaction mixture. As required, extracts of bacterial cultures were chromatographed for detection of IAA or tryptophan. Ascending chromatograms were made on Whatman No. 1 filter paper by using the following solvent: isopropanol-8 N NH_4OH (4: 1 v/v). Chromatograms were sprayed with Salkowski reagent to develop color.

Nitrite was determined by a modified Shinn (17) technique, 1 ml of 1 % sulfanilic acid in 1.5 N HCl and 1 ml of 0.02 % N (1-naphthyl)-ethylenediamine • 2HCl in water were added to a 2 ml sample of the solution under study and the optical density was measured at 525 mμ. The density values were converted to molar concentrations from a standard curve and reported as μmoles/ml of reaction mixture.

Reaction mixtures were diluted 10-fold for tryptophan assay using a tryptophan-requiring mutant of *Lactobacillus arabinosus* in the standard tryptophan bioassay described in the Difco Manual (4).

Results

Rhizobial Production of IAA. The conversion of tryptophan to IAA by 4 strains of rhizobia was measured in the absence of added nitrogen. Because the density of the cells in the reaction mixtures varied somewhat between experiments, there were some differences in IAA production between experiments. Strain 117 was very slow in producing IAA, and IAA could not be detected in some experiments. Strains Su 388, 205 and 123 produced IAA rapidly and in relatively high concentration. Figure 1 illustrates results typical of this group of experiments.

Nitrate Reduction by Rhizobia and IAA Destruction by the Nitrite Produced. The motile form of rhizobia reduced nitrate to nitrite as had been reported with the bacteroid or nodular form (3). The reduction of nitrate to nitrite by rhizobia varied greatly between experiments even though care was taken to provide uniform conditions throughout. The reason for this variability was not determined.

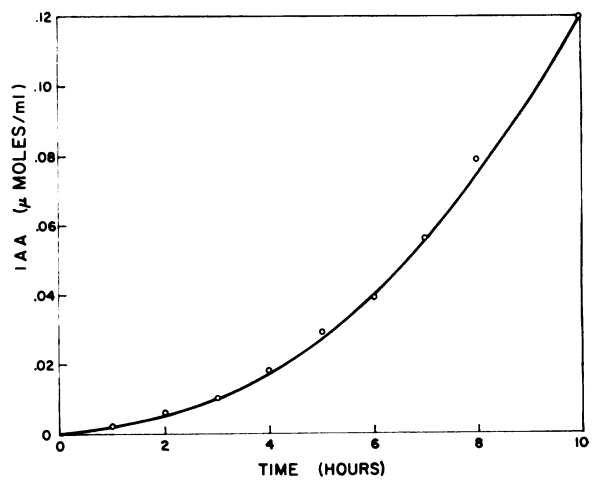

FIG. 1. Conversion of tryptophan to IAA by rhizobia. Strain Su 388 was used in a reaction mixture containing 10^{-3} M tryptophan.

Supernatant liquids, taken from reaction mixtures containing strain 117 and 10^{-2} M nitrate, destroyed added IAA. A 2-ml sample of supernatant liquid when removed after 1 hour of incubation contained 0.004 μmole of nitrite. Two ml of a solution containing 2 μmoles of IAA were added to the cell-free sample. After 11 hours, 1.92 of the 2 μmoles of IAA had been destroyed. These results demonstrate the catalytic action of nitrite. Boiling of the supernatant liquids did not reduce their ability to destroy IAA. Samples taken at zero time did not cause destruction of added IAA.

The water layer remaining after IAA had been extracted twice with ether appeared visibly orange-brown. This would approximate the tan precipitate that Tonhazy and Pelczar (25) suspected to be polymerized indole-3-aldehyde, which was produced from IAA by nitrite. Both strains of *R. japonicum* (117 and 123) caused a more rapid and greater accumulation of nitrite than did the *R. meliloti* strain (Su 388). In experiments where no nitrite was produced, there was no destruction of IAA.

Simultaneous Production of IAA and Nitrite by Mixed Strains of Rhizobia. Preliminary experiments had shown that strain 117 produced relatively large amounts of nitrite from nitrate. Strains Su 388 and 205 showed a greater tendency to convert tryptophan to IAA. A reaction mixture containing strain 117 and either Su 388 or 205 would conceivably produce a system in which both reactions would occur strongly. The following reaction mixtures were set up with mixed strains of rhizobia: tryptophan, tryptophan + nitrate, and tryptophan + ammonium. Experiments were carried out by using a reaction mixture containing strains Su 388 and 117 and by using a mixture containing strains 205 and 117.

The results obtained by mixing strains showed both nitrite and IAA production and IAA destruction by nitrite (table I). Where nitrite was produced, some of the IAA was destroyed, thereby yielding a lower IAA concentration than with no nitrate added. Additions of ammonium reduced final concentration of IAA with no production of nitrite, indicating that something other than nitrite was lowering the final concentration of IAA.

Inhibition of IAA Formation by Reduced Nitrogen Sources. Previous experiments had indicated that considerably less IAA was formed when ammonium was present in the reaction mixture. An experiment was set up to quantitatively determine the effect of levels of ammonium on IAA production (table II). As concentration of ammonium increased, less IAA was formed. Decreased formation of IAA was not due to destruction by nitrite. It appeared, therefore, that, in the presence of ammonium, less tryptophan was being converted to IAA. A series of experi-

Table I. *Effects of Nitrate and Ammonium on IAA Content of Reaction Mixtures Containing 2 Strains of Rhizobia*

Strains	Reaction time (hr)	Reaction mixture*		
		Control	+ KNO_3	+ NH_4Cl
		μmole IAA/ml		
388 + 117	16	0.013	0.000	0.007
388 + 117	24	0.054	0.015	0.019
205 + 117	12	0.063	0.021	0.022
205 + 117	20	0.089	0.027	0.017
	Average	0.055	0.016	0.016
		μmoles NO_2^-/ml		
388 + 117	16	0	6.3	0
388 + 117	24	0	0.1	0
205 + 117	12	0	10.0	0
205 + 117	20	0	4.0	0

* Concentrations of KNO_3 and NH_4Cl were 10^{-2} M, the concentration of tryptophan was 10^{-3} M, and approximately 0.5×10^6 cells of each strain were used per ml.

Table II. *Effect of Ammonium Concentration on Rhizobial Conversion of Tryptophan to IAA*

A mixed culture of strains 117 and Su 388 was used. The mixtures were shaken for 12 hours.

NH_4Cl (M)	IAA produced (μmoles/ml)	Nitrite produced (μmoles/ml)
0	0.136	0
3.33×10^{-3}	0.033	0
10^{-2}	0.023	0
2×10^{-2}	0.019	0
4×10^{-2}	0.015	0

ments was set up to test this hypothesis. Various sources of nitrogen were used, and analyses were made for IAA, nitrite, and residual tryptophan (table III). All sources of nitrogen reduced the level of IAA. Nitrate did not appreciably decrease utilization of tryptophan by rhizobia but resulted in the presence of low amounts of IAA. The other nitrogen sources reduced the utilization of tryptophan as evidenced by the greater amounts of residual tryptophan. All reduced nitrogen sources depressed the production of IAA. In most of the reaction mixtures approximately 0.25 μmole of L-tryptophan was not recovered. Presumably this was incorporated into the cells, although it may have undergone other reactions which were not detected.

Discussion

Evidence in the literature supports a local (external) effect of combined nitrogen on inhibition of nodulation. Although the precise role of IAA is not known we have assumed its essentiality for rhizobial infection and nodule initiation in conducting this study and proposing a mechanism. Assuming that IAA is essential for infection, it follows that substances which reduces IAA concentration below a certain critical level would reduce and/or delay infection. The duration and severity of the inhibition would be proportional to the concentration of the inhibiting material.

Previous work (19) had indicated that essentially the same amount of tryptophan was converted to IAA with and without added nitrate. The tan precipitate present in the water fraction after the IAA had been extracted with ether was evidently the same as that which Tonhazy and Pelczar (25) proposed to be polymerized indole aldehyde. In the presence of nitrate, IAA production was not prevented; the observed decrease in IAA resulted from nitrite destruction of IAA.

The lower level of IAA in the presence of ammonium, urea, or glycine, was the result of a different mechanism. Tryptophan conversion to IAA occurred at a greatly reduced rate in the presence of reduced nitrogen sources. This was shown by determining residual tryptophan after cells were incubated with and without these reduced forms of nitrogen in the tryptophan media.

Using the preceding observations, mechanisms for a local (external) effect of combined nitrogen or nodulation are proposed (fig 2). Reduced concentration of IAA is essential to both mechanisms, although the means by which this is achieved differs with nitrate and the reduced forms of nitrogen.

Table III. *Rhizobial Production of IAA from Tryptophan and Residual Tryptophan in the Presence of Several Nitrogen Sources*

Strain 117 was used with a reaction time of 24 hours. The molar concentrations of the nitrogen source was 10^{-2}. The initial concentration of L-tryptophan was 0.5 μmoles/ml.

Reaction mixture	IAA found (μmoles/ml)	Nitrite produced (μmoles/ml)	Residual tryptophan (μmoles/ml)
Tryptophan	0.218	0.0	0.008
Tryptophan + KNO_3	0.027	0.2	0.050
Tryptophan + NH_4Cl	0.023	0.0	0.168
Tryptophan + urea	0.040	0.0	0.200
Tryptophan + glycine	0.049	0.0	0.205

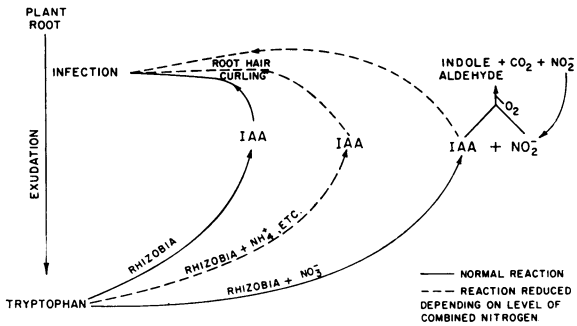

FIG. 2. Diagram of proposed mechanisms for the inhibition of rhizobial infection by various forms of nitrogen.

Concentration of combined nitrogen would determine the duration of the delay or inhibition of infection. Complete inhibition would be expected where IAA concentration reached a certain minimum level. Concentrations of IAA in extracts of root media are of doubtful significance (9) and probably differ markedly from actual rhizosphere concentrations.

The observed differences between strains of rhizobia in ability to reduce nitrate and in ability to convert tryptophan to IAA could be factors influencing virulence.

In postulating an external mechanism for nitrogen inhibition of nodulation, the problem of the relative importance of the external versus the internal effect is naturally raised. Their relative importance is difficult to assess. Under normal conditions both are probably operative. However, the internal C: N ratio primarily affects nodule initiation and development; the external mechanisms postulated retard or inhibit infection. Infection must chronologically precede nodule initiation and development; hence, the external mechanisms could preclude the internal effect.

Because of the heterogeneous nature of the rhizosphere, isolated "escapes" probably do gain entrance under high soil nitrogen. In such instances, and when rhizobia gain entrance via wounds, the internal C: N ratio would inhibit nodule initiation and development. The 2 theories (internal and external) would not appear contradictory but, instead quite complementary and compatible.

Summary

Data have been presented in support of the hypothesis that combined nitrogen exerts its inhibition of nodulation by reducing the external concentration of indoleacetic acid. Rhizobia reduced nitrate to nitrite. Nitrite was capable of catalytically destroying indoleacetic acid. Ammonium, urea or glycine also reduced the formation of indoleacetic acid, but not through the formation of nitrite.

Rhizobial species and strains differed in their abilities to reduce nitrate to nitrite and in converting tryptophan to indoleacetic acid.

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Reversible Changes in the Hydraulic Permeability of Plant Cell Membranes [1, 2]

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Introduction

In comparison with the numerous studies into factors affecting the permeability of tissues to electrolytes, nonelectrolytes, and dyes (4), studies on changes in permeability to water have been very few in number. Moreover, much of the reported work has been based on measurements of the rates of plasmolysis and deplasmolysis (5, 7, 15). According to Myers (10) the permeability of plasmolysed cells to water is completely different from that of cells which have not been plasmolysed.

Another important criticism can be leveled at all the former work. Treatment of osmotic water flow according to the principles of irreversible thermodynamics has focused attention on a second parameter which is of equal importance with hydraulic permeability in defining water flux through the membrane (17, 8, 3). This second parameter is σ, the reflection coefficient. Its derivation and its importance in botanical studies have recently been lucidly explained by Dainty (2). This coefficient is only equal to 1 where the membrane is ideally semipermeable, or where no interaction occurs between solute and solvent as they pass through the membrane. As will be demonstrated below, effects that have been attributed to changes in permeability to water may have been caused by changes in σ. None of the earlier work on factors affecting permeability has taken this parameter into account.

Following our recent observation (6) that CO_2 brought about a rapid change in rate of water movement into and out of plant cells, we wished to determine whether this effect was brought about via a change in σ or in L_p, the coefficient of hydraulic permeability. The distinction is of considerable qualitative importance; whereas a drop in L_p indicates a decrease in permeability to water, a drop in σ would imply an increase in permeability to solutes.

To examine this question we have had to abandon the classical equation for water uptake into plant cells based on the concept of Diffusion Pressure Deficit. Apart from its other serious defects [recently criticized by Slatyer and Taylor (16) and Ray (13)] it is inadequate for dealing with water flow when the membrane is permeable to the solutes as well as to the solvent (2). We have adopted the equation based

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